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Novel set of groundnut SSR markers for germplasm analysis and interspecific transferability

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Abstract
Cultivated groundnut (*Arachis hypogaea* L.), is an important oil seed crop grown mostly in tropical and subtropical regions of the world. However, it suffers with narrow genetic diversity due to genetic bottlenecks such as the recent polyploidization and self pollinating nature of the crop. In such crop species, simple sequence repeats (SSRs), as compared to other marker systems such as restriction fragment length polymorphisms (RFLPs), randomly amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) have been found more informative for genetic studies and breeding applications. Therefore, with an aim to develop new SSR markers in groundnut, a SSR-enriched library was constructed from the genotype ICGV 86031. Out of 29 SSRs isolated, primer pairs were designed for 23 SSR loci, of which 14 (61%) primer pairs yielded scorable amplicons. Eight (57%) primer pairs showed polymorphism among 23 groundnut genotypes that are parents of different groundnut mapping populations at ICRISAT, India and EMBRAPA, Brazil. The polymorphism information content (PIC) for polymorphic SSR markers ranged from 0.13 to 0.36, with an average of 0.25. Newly isolated SSR loci showed good interspecific transferability rates ranging from 13% to 100% across seven legumes and 43% to 100% within the seven legumes. The present set of newly developed SSR markers enriches the existing groundnut SSR repertoire and the transferable SSR markers will be useful for comparative genome analysis in related legumes.

Keywords: Groundnut, microsatellite, polymorphism, transferability, genetic diversity.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.), also known as peanut, is a valuable cash crop for millions of small-scale farmers living mostly in the semi-arid tropics. It is native to South America and belongs to the legume family *Fabaceae.* The genus *Arachis* has 80 species described (Valls *et al*., 1994), nearly all *Arachis* species are wild and diploid ($2n=2x=20$), but the cultivated groundnut *Arachis hypogaea* is an allotetraploid (AABB) ($2n=4x=40$). Cultivated groundnut is an important source of edible oil and proteins. It is grown through out the world in over 100 countries with a total area of 25.2 M ha and 36.5 M tons of global production in FAO statistical database (http://fastat.fao.org/, 2006), India, China and USA are the largest producers.

Although, crop improvement of groundnut has been undertaken by conventional breeding, no effective molecular breeding programme has been initiated to facilitate genetic improvement in this crop (Varshney *et al*., 2007). In contrast to the diversified variations observed in morphological traits of cultivated groundnut, its genetic variations at molecular level as detected by RAPD (randomly amplified polymorphic DNAs) and RFLPs (restriction fragment length polymorphisms) were unexpectedly low (Halward *et al*., 1993). Reasons for the low level of genetic variation observed in cultivated groundnut can be attributed to (i)

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barriers to the gene flow from related diploid species to domesticated groundnut as a consequence of the polyploidization event (Young *et al*., 1996), (ii) recent polyploidization from one or a few individuals of each diploid parental species, combined with self pollination, (iii) narrow genetic base of cultivated germplasm due to use of few elite breeding lines and little exotic germplasm in breeding programs and (iv) unavailability of larger and more suitable molecular markers (Varshney *et al*., 2007). However, through the use of appropriate genomic tools genetic variation present in the primary gene pool of this crop can be harnessed for crop improvement programmes (Varshney *et al*., 2009a). Indeed, in several crop species, mainly temperate cereals, genomic tools and approaches have already shown their utility in genetic analysis and breeding (Varshney *et al*., 2005a; Varshney *et al*., 2006).

Microsatellites, also known as simple sequence repeats (SSRs), are tandem array of nucleotides (one to six) spread all over the genome. Microsatellites have become the markers of choice in recent past due to their higher information content and other features such as high reproducibility and co-dominant nature (Gupta and Varshney, 2000) Microsatellites have other comparative advantages over other DNA markers, e.g., they are multi-allelic, highly abundant, analytically simple and readily transferable. As a result, the SSR markers have been found more useful than RFLPs and RAPDs, and have been widely utilized in plant genetic studies (Gupta and Varshney, 2000). In the case of groundnut, and as a result of considerable efforts of several researchers, several hundred SSR markers have become available (Varshney *et al*., 2007). These SSR markers have been found very useful to detect genetic variability in groundnut germplasm (Cuc *et al*., 2008; Varshney *et al*., 2009a) and also in the construction of genetic linkage maps for diploid (Moretzsohn *et al*., 2005; Moretzsohn *et al*., 2009) and tetraploid genomes (Varshney *et al*., 2009b) of groundnut.

In view of the importance of SSR markers in groundnut, the present study was undertaken with the following objectives: (i) to isolate new SSR markers for extending the groundnut SSR repertoire, (ii) to assess the potential of newly developed SSR markers for detection of polymorphism in groundnut accessions, and (iii) to test the inter-specific transferability of novel SSRs developed to seven related legumes namely pigeonpea, cowpea, soybean, common bean, black gram, chickpea and Medicago.

MATERIALS AND METHODS

Plant material and DNA extraction

For construction of SSR enriched library, the groundnut germplasm line ICGV 86031 was used. While two genotypes ICGV 86031 and TAG 24 were used initially for optimizing the PCR assays for newly developed SSR markers, another set of 23 genotypes consisting of cultivated varieties, synthetic tetraploids and wild groundnut genotypes was used to assess the potential of new SSR markers developed (Table 1 *[Supplementary data]*). These include parental genotypes of different cultivated and wild groundnut mapping populations. For testing the inter-specific transferability of newly developed SSR markers seven legumes of the subfamily Papilionoideae namely pigeonpea (*Cajanus cajan*, ICPL 87119), cowpea (*Vigna ungiculata*, Arka suman), soybean (*Glycine max*, J335), common bean (*Phaseolus vulgaris*, Arka suvidha), blackgram (*Vigna mungo*, LBG 623), chickpea (*Cicer arietinum*, ICC 4958) and Medicago (*Medicago truncatula*, Mt A17) were used.

Total genomic DNA for above mentioned genotypes was either isolated from unfurled leaves and purified according to a modified CTAB-based protocol (Cuc *et al*., 2008) or procured from collaborators.

Construction of SSR enriched library

A new SSR enriched library was developed from the groundnut genotype ICGV 86031 using bead capture enrichment protocol by Glenn (Glenn *et al*., 2005). Total genomic DNA was digested with *Rsa*I and *Xmn*I (New England BioLabs, UK) and restriction fragments were ligated into the double stranded super SNX24 (5'GTTTAAGGCCTAGCTAGCAGAATC) and super SNX 24+4p (5'pGATTCTGCTAGCTAGGCCTTAAACAAAA) adapters. The library was enriched with five biotinylated oligonucleotides $(CT)_{10}$ - 42°C, $(TG)_{12}$ - 45°C, $(AG)_{14}$ - 45°C and $(AAG)_8$ - 40°C using Streptavidin M-280 Dynabeads, (Dynal, Oslo, Norway). The enriched DNA fragments were further amplified using Super SNX 24 forward primer (30 cycles with 60°C annealing temperature). Subsequently SSR enriched products were ligated into pCR2.1-TOPO vector (Invitrogen, Carlsbad, USA) and further transformed into *Escherichia coli* competent cells (TOP 10, Invitrogen, Carlsbad, USA) plated on LB agar containing ampicillin, X-gal and IPTG to allow for blue white colony selection. Subsequently, the white colonies were picked and grown overnight in 2 ml of LB broth with 100 µg/ml ampicillin. Plasmid DNA was extracted using alkaline lysis method and the plasmid DNA was sequenced using M13 Forward 19-mer sequencing primer following the dideoxynucleotide chain termination method on ABI 3700 sequencer.

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SSR identification and primer designing

Raw sequence data were further trimmed using a sliding window of 50 bp with a minimum average Phred score of 20 and filtered length of 100 bp. Sequence data were searched with *Mi*cro*SA*tellite (*MISA*) Perl script for mining and identification of SSRs, and primer pairs for flanking regions of SSR were designed using Primer 3 (Primer3).

Polymerase chain reaction and fragment analysis

PCR reactions for all the primer pairs were performed in a 5 ul reaction volume following a touchdown PCR profile in an ABI 9700 thermal cycler (Applied Biosystems, Foster city, USA). The PCR reactions were performed using ~5ng of genomic DNA with 1 picomole of forward primer anchored with M13 tail, 2 picomoles of unlabelled reverse primer and 2 picomoles of M13 fluorescent label (6-FAM), 2 mM of each dNTP, 2 mM $MgCl_2$, 1X amplification buffer and 0.2 U of *Taq* DNA polymerase. Touchdown PCR amplification profile has initial denaturation step for 3 min followed by first 5 cycles of 94°C for 20 sec, 60°C for 20 sec and 72°C for 30 sec, with 1°C decrease in temperature per each cycle, then followed by 35 cycles of 94°C for 20 sec with constant annealing temperature (56°C) and 72°C for 30 sec, followed by a final extension for 20 min at 72°C. Amplification products were resolved on capillary electrophoresis system, ABI 3130 Genetic Analyzer. Fragment analysis was done using Genescan and Genotyper software version 3.7 (Applied Biosystems, Foster city, USA).

Polymorphism detection

Allelic data of the scorable markers was subjected to AlleloBin software (Chandra *et al.*, Unpublished) to obtain allele calls based on repeat motif and number of alleles detected by each SSR markers was counted.

Polymorphic information content (PIC) for different markers was calculated using POWERMARKER v 3.25 software (Liu *et al*., 2005).

The allelic data obtained from the polymorphic markers was converted to binary data (0/1). The 0/1 binary data of the markers was used for the calculation of genetic distances and an UPGMA (Unweighted Pair Group Method with Arithmetic mean) dendrogram was constructed using PAUP* 4.0b10 (Swofford, 1998) and Dendroscope (Huson *et al*., 2007).

Cross species transferability

For checking transferability of novel SSR markers developed in seven legumes, the PCR conditions were kept same as were used for amplification of SSR loci in groundnut. Transferability data were collected as presence or absence of band at the locus amplified by the particular marker. Presence of band was scored as (+) and absence of band was scored as (-). Percentage transferability was recorded as percentage of amplification of the SSR markers amplified in seven legumes tested.

Single and strong amplicons generated by the primer pair ICGM01A11c across the seven legumes were sequenced using BDT v 3.1 cycle sequencing kit (Applied Biosystems, Foster city, USA) on ABI 3130 genetic analyzer and the resulting sequences were checked for sequence similarity by multiple sequence alignment using sequence alignment editor in Bioedit Programme v 7.0.5.3.

RESULTS

Isolation of SSRs and marker development

SSR enriched library was constructed from the genotype ICGV 86031 following the method of Glenn and Schable 2005. The library was enriched for (CT), (TG), (AG) and (AAG) repeat motifs. In the first instance a total of 72 positive clones were selected for isolation of plasmid DNA. The insert size in these clones was estimated in the range of 100 to 300 bp. Sequencing of plasmid DNA for these 72 positive clones resulted in good quality sequences in 65 cases. Sequence data for these 65 clones were submitted to Genbank under accession numbers FI857100 to FI857164.

Analysis of sequence data for 65 clones with the *MISA* perlscript showed the presence of one or more SSRs in 29 clones (45%). Following the definition of Weber (1990), 61% of SSRs identified were perfect, 4% were imperfect and 35% were compound repeats. While twelve SSRs identified contained tetra-nucleotide repeats (52%), nine (39%) had compound repeats, one had tri-nucleotide repeat (4%) and the other one contained penta-nucleotide repeat (4%). In terms of frequency or abundance of a particular SSR, the CTAG repeat motif was found more abundant (30%). Since the number of SSRs isolated in this study is low, results based on this study may not be of much significance to speculate the frequency and abundance of SSRs in the groundnut genome.

For developing the new markers based on SSRs isolated, designing of primer pairs was attempted for all 29 sequences. The primer pairs however could be designed for only 23 sequences (35%) (Table 2 *[Supplementary data]*). In the remaining cases, sequences flanking SSR regions were too short to design primers. The newly developed SSR markers were designated as

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ICGM (ICRISAT Groundnut Microsatellite followed by clone ID). For testing the amplification of these new SSR markers, two groundnut genotypes namely ICGV 86031 and TAG 24 were used for PCR amplification. As a result, only 14 (61%) primer pairs amplified scorable amplicons (Table 2).

Polymorphism assessment of novel SSRs

The set of 14 primer pairs identified above were used to screen polymorphism in 23 diverse cultivated and wild groundnut lines. Of these markers, only 8 (57%) markers showed polymorphism among 23 lines while the remaining 6 markers were found monomorphic. The polymorphic markers (8) amplified a total of 18 alleles with an average of 2.3 alleles per locus. The PIC values ranged from 0.13 (ICGM01A04b) to 0.36 (ICGM01A11c) with an average of 0.25. Highest PIC value was observed with primer pair ICGM01A11c (0.36) followed by ICGM01A05a and ICGM01A12c (0.35) and the least PIC value was observed with the primer pair ICGM01A04b (0.13) (Table 3).

Table 3: Polymorphism study of novel set of SSR markers.

Marker ID	No. of Alleles	PIC value
ICGM01A04b	2	0.13
ICGM01A05a	2	0.35
ICGM01A05b	2	0.26
ICGM01A10b	3	0.22
ICGM01A11b	2	0.20
ICGM01A11c	2	0.36
ICGM01A12b	2	0.16
ICGM01A12c	3	0.35
Mean	**2.3**	**0.25**

SSR transferability in legumes

The SSR markers developed in the present study were used for examining their interspecific-transferability in the selected seven legumes: pigeonpea, cowpea, soybean, common bean, blackgram, chickpea and Medicago. The percentage interspecific-transferability of all newly developed SSR markers ranged from 43% (chickpea) to 71% (cowpea, common bean and Medicago) (Table 4 *[Supplementary data]*) across species examined. In terms of percentage transferability of individual SSR markers, ICGM01A05a showed 13%, ICGM01B04a and ICGM01A03a showed 25%, ICGM01A08c and ICGM01A04b showed 50%, ICGM01A06c and ICGM01A11b showed 63%, ICGM01A05b, ICGM01A06b, ICGM01A10b, ICGM01A12b and ICGM01A12c showed 88%, and ICGM01A11c and ICGM01B03a showed 100% transferability to the seven legumes tested. However, one primer pair ICGM01A05a did not amplify in any of the seven legumes tested.

In order to validate the amplicons generated by the new set of SSR markers in different legumes, amplicons generated in all the seven legumes by one marker ICGM01A11c were sequenced for checking the conservation of repeat motif. Sequence information revealed that the SSR motif $(AAAG)_2$ of the marker ICGM01A11c of groundnut is present in all the legumes studied.

Genetic relationships in Arachis

The *Arachis* germplasm evaluated with the new set of SSR markers consisted of 17 cultivated tetraploid genotypes ($2n=4x=40$), three synthetic amphidiploids ($2n=4x=40$) and three wild diploid genotypes ($2n=2x=20$). A total of 18 alleles amplified by eight polymorphic markers were used for construction of dendrogram to understand the genetic relationships among the *Arachis* germplasm. The resulting dendrogram classified the *Arachis* germplasm into four main clusters (Fig. 1). Cluster I and Cluster II are exclusively formed by cultivated tetraploid accessions, Cluster III is exclusive to amphidiploids and Cluster IV is exclusive to the wild (diploid) accessions. Cluster I is composed of eight cultivated tetraploid accessions from India except IAC-R886 which is a Brazilian accession. Cluster II is also composed of nine cultivated tetraploid accessions from Brazil and Japan with exceptions ICG 7827 and TMV 2 which are accessions from India. Cluster III is exclusively formed by the three amphidiploids used in this study TxAG 6, V 14167×K 30076 and V 9401×V 6389. Cluster IV contained the two wild accessions K7988 and V10309 but the other wild accession K30097 has come out as a separate out-group closer to cluster IV which has the other two wild accessions. In brief, the genotypes analyzed in this study were grouped mainly according to their ploidy level and geographic origin.

DISCUSSION

Novel SSR markers for groundnut genetics and breeding

Microsatellites have become the most widely used molecular markers for genetic studies in recent years. Construction and screening of partial genomic libraries and sequencing of SSR positive clones have been found to be an effective method for SSR isolation (Rafalski *et al*., 1996). Enrichment of genomic DNA libraries for SSRs enhances the SSR isolation efficiency (Edwards *et al*., 1996). Therefore, to isolate new SSR markers for groundnut, a SSR enriched library was constructed in the present study. Out of the 65 positive clones sequenced 29 clones had unique SSRs (45%) and primer pairs could be designed for 23 SSR containing sequences (35%). Even though a lesser number of SSR-positive clones were sequenced in the present study, the results obtained are comparable to the earlier groundnut

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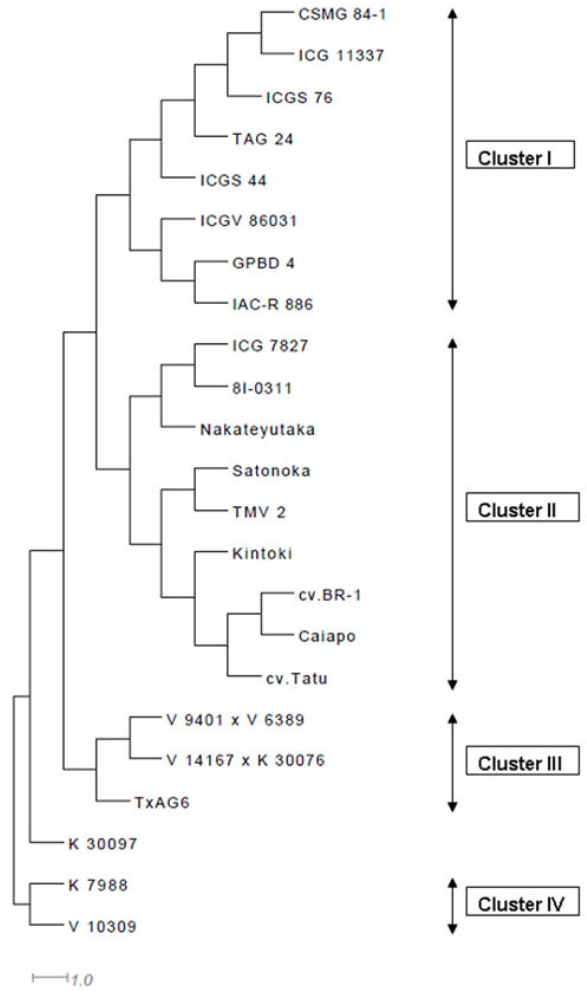

Figure 1: Cluster analysis of 23 groundnut accessions based on genotyping data of 8 polymorphic SSR markers amplifying 18 alleles .

SSR isolation studies. In case of Hopkins *et al*., (1999), 66 (55%) out of the 120 sequenced "positive" clones had SSRs, but primer pairs could be designed for only 26 (22%). Gao *et al*., (2003) identified 14 (6%) unique SSR-containing sequences in 256 clones. Similarly He *et al*., (2003) sequenced 401 randomly picked clones resulting from AFLP pre-amplification based protocol, of which 83 (21%) were unique SSRs and primer pairs were designed for 56 (14%). Ferguson *et al*., (2004) identified 348 (21%) SSRs by sequencing 1,627 clones, merely 226 (14%) primers could be designed. The SSR enrichment efficiency depends on many factors including the choice of restriction enzyme used for library construction, the SSR probes used for enrichment and optimization of PCR profile and conditions. The approach used in the present study appears to be considerably efficient enrichment strategy for SSR isolation in groundnut.

In the current study all the SSRs identified had different repeat motifs that were not totally complementary to the sequences of oligonucleotide probes used for library enrichment. Earlier Gimenes (Gimenes *et al*., 2007) also observed that 37% of SSRs isolated had a different repeat motif. The ATT repeat motif which is considered most abundant and highly informative in several legumes like soybean (Akkaya *et al*., 1992), chickpea (Huttel *et al*., 1999) and pea (Burstin *et al*., 2001) was not observed in the present study. These observations can be explained by the reason that the total number of SSR positive clones sequenced in the present study is far lower than the earlier studies.

In terms of converting SSRs into informative markers for groundnut genetics and breeding, validation of 23 SSR markers on two genotypes provided 14 useful markers which showed amplification. Polymorphism assessment of these 14 markers on 23 cultivated, synthetic and wild *Arachis* genotypes revealed polymorphism incase of eight markers (57%) with at least one of the 23 genotypes. The percentage polymorphism observed in the present study is higher than in other studies by He (He *et al*., 2003) (33%), Ferguson (Ferguson *et al*., 2004) (28%) as the present study, unlike the mentioned studies, employed screening of synthetic and wild genotypes along with cultivated genotypes. The average number of alleles (2.3) and PIC values (0.25) observed in the present study are comparatively lower than the earlier studies. In the case of Moretzsohn (Moretzsohn *et al*., 2004), the average number of alleles observed was 5.3 and average PIC value was 0.56. This could be explained by the fact that a larger number of accessions (60) was used compared to the present study where only 23 accessions were used to test polymorphism. In general, the SSR markers developed in this study enhances the existing repertoire of SSR markers in groundnut and should be useful for genetic analysis and breeding applications in groundnut.

Transferability of novel SSR markers

Transferability of SSR markers to the related genera is a consequence of the homology of flanking sequences of the microsatellites and size of the region between the primer pairs amenable to amplification by PCR. Earlier studies have demonstrated the conservation of SSR sequences of one plant species in the related plant species (Gupta and Varshney 2000; Varshney *et al*., 2005a; Varshney *et al*., 2005b). The donor source of primer pairs influences levels of transferability, which reflects the genetic distance between the donor and target species. Apart from this, other factors such as mutations in flanking regions, ploidy level (Dirlewanger *et al*., 2002), large intronic regions in case of genic SSRs, template DNA concentration and PCR conditions used may complicate the relationship of transferability.

In the present study a total of 14 SSR primer pairs were studied to test their transferability rates to seven

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legumes. The percentage transferability of these primer (ICGM01A11c and ICGM01B03a) across the seven legumes and 43% (chickpea) to 100% (groundnut) within the seven legumes tested (Table 4). Inter-specific transferability of SSR markers was observed in several other studies (Peakall *et al*., 1998; Wang *et al*., 2004) that can be explained by the reason that the flanking regions of these SSRs are conserved across different legumes. The primer pair ICGM01A05a did not amplify in all the seven legumes tested which may be due to absence of the particular locus in the seven species and is unique to groundnut or there may be a possibility of mutations in the flanking regions in the form of insertions or deletions. The SSR markers designed for groundnut in the present study may be used to detect polymorphism in related species like pigeonpea and mung bean where limited numbers of SSR markers are available.

Earlier studies have reported homoplasy where the amplicons generated by same SSR primer pair in different species are of similar length but differ in their sequence (Varshney *et al*., 2005a) which may be due to mutations in the SSR containing region and at the same time the flanking regions of SSR are conserved. In order to confirm the amplification of similar SSR locus in other species, for which the primer pairs were designed in groundnut, sequence analysis of amplicons obtained in seven legumes for one marker ICGM01A11c showed the conservation of SSR motif $(AAAG)_2$ across all the legumes tested. Based on extrapolation of results of one marker for sequence conservation, it may be inferred that repeat motives of majority of groundnut SSRs developed are conserved in different legumes tested.

Grouping and genetic diversity

Knowledge and management of genetic diversity are critical for any crop improvement program. In crops like groundnut with a narrow genetic base, diversity studies to characterize different accessions and for identifying diverse parental lines for developing mapping populations assume much importance. The tree dendrogram prepared based on the genetic diversity data showed grouping of the genotypes into four clusters based on their ploidy level or geographic origin. In the present study even though a very limited number of markers (8 markers amplifying 18 alleles) were used for analysis, they were able to differentiate the cultivated tetraploid accessions, synthetic amphidiploids and wild diploid accessions into separate groups. It indicates the potential of SSR markers isolated for genome relationship and genetic diversity studies. Some accessions from India (TMV 2 and ICG 7827) were grouped along with accessions from Japan and one accession from Brazil (IAC-R886) was grouped with Indian accessions. This kind of grouping may be due to probable admixture of alleles between pairs ranged from 13% (ICGM01A05a) to 100% these accessions from different geographic regions, use of more number of markers may clearly differentiate the accessions based on their geographic origin.

In summary, the present study adds a set of 14 new novel SSR markers, to the existing groundnut SSR repertoire. It is desirable to isolate and characterize more DNA markers in cultivated groundnut for genomic studies like genetic mapping, marker assisted selection and gene discovery. The transferable SSR loci identified in the present study can be of use in the genetic analyses of the seven legumes.

Abbreviations

SSRs: Simple sequence repeats
PIC: Polymorphism information content
X-gal: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
IPTG: isopropyl-beta-thio galactopyranoside
LB: Luria Broth
MISA: *Mi*cro*SA*tellite Perl script
6-FAM: 6-Carboxyfluorescein
ICGM: ICRISAT Groundnut Microsatellite
PAUP*: Phylogenetic analysis using parsimony

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